



## Original Research

# Reevaluating the Sperm Nuclear Chromatin Decondensation Test by Sodium Thioglycolate of Stallions Spermatozoa



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## ARTICLE INFO

## Article history:

Received 8 June 2015

Received in revised form

18 September 2015

Accepted 12 October 2015

Available online 23 October 2015

## Keywords:

Stallion

Sperm nucleus

Chromatin compaction

Sodium thioglycolate

## ABSTRACT

The nucleus of mammalian spermatozoa is mainly composed of chromatin associated with protamines: highly basic proteins (around 7 kDa). These highly basic proteins, due to their cysteine content, can participate in the generation of disulfide bond. These characteristics permit typical condensed nuclear structure in mature spermatozoa, where the DNA becomes organized in compacted units, similar to nucleosomes, but with 60 kb of DNA. This shape is ultimately assumed in the epididymal maturation, and the level of compaction is closely related to epididymal function. In the present work, we present a modified method to evaluate sperm decompaction using sodium thioglycolate (ST). Stallion sperm were exposed to different ST concentrations and were embedded in agarose as a supportive medium. With the use of agarose, it was easier to identify patterns of decompaction with ST, and, thus, the use of a permeabilizing solution was not necessary. This was due to the utilization of ST to evaluate chromatin compaction of stallion sperm physically permeabilized and embedded in agarose matrix.

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## 1. Introduction

In the seminiferous tubule of testis, where spermiogenesis occurs, spermatids nuclei undergo complex morphologic, biochemical, and physiological changes to complete their development. One of these changes is related to nuclei condensation due to the exchange of histones primarily by transition proteins, after highly basic arginine and cysteine rich protamines. Later, the nucleus will be composed of highly compacted chromatin during the epididymal transit because of the formation of

disulphide bonds between free sulfhydryls on cysteine of protamines that will be oxidized to form disulphide bonds [1]. This produces protamine-DNA chromatin, generating tightly packed toroidal-like structures, containing up to 60 kb of DNA, in which the transcription and repair activities are inactivated and also related to morphology of sperm head and maturation state of sperm during epididymal transit [2].

As one of the features that can provide functional information of the sperm, several approaches for assessing the presence of disulfide bonds have been tested involving reductant agents such as including dithiothreitol (DTT) + sodium dodecyl sulfate (SDS) detergent, and sodium thioglycolate (ST) among others [3,4]; being their effect in the sperm nucleus mainly evaluated as changes in sperm head size.

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Sodium thioglycolate was chosen because, as was published by Fornés and Bustos-Obregón [5], the fact that it could freely pass the plasma membrane. In stallion sperm, ST tests have been used to study compaction level [6]. However, their observation and evaluation is not easy, because mainly to a weak and nonspecific staining, heterogeneous response of sperm to decondensation process, that evidenced it with several patterns of staining (color and size of halo). Moreover, to date, there is no evidence that the sodium thioglycolate across freely and without resistance through the plasma membrane of the sperm. If not the case, part of then response to decondensation depends on if the sodium thioglycolate effectively was incorporated to citosol and later reacting with sperm chromatin, aspect that was evaluated in this work.

Here, we propose the use of a new protocol of ST as a reducing agent for disulphide bonds, overcoming the lack of the effect in sperm with plasma membrane integrity, whereas with the original protocol, only sperm with their membrane disrupted presented reactive characteristics. This differs from the work of Fornés and Bustos-Obregón [5], in which all cells are expected to react with ST, and owing to the fact that the conditions in which the sperm cells are immobilized and attached to the slide for observation are different, being the agarose a supportive medium for controlled and homogeneous sperm sample decompaction.

## 2. Materials and Methods

### 2.1. Sample Preparation and Selection

In this study, only frozen semen samples were used. The samples were obtained from ejaculates of five stallions, with fertility probed with frozen semen, and with ages between 7 and 16 years old, from Heavy Draft breeds, property of Haras Militar Pupunahue (a member of the Fondef D0811076 project), which explicitly approved the use of the samples for scientific research purposes.

Qualified samples were cryopreserved according to standard procedure, using a BotuCrio freezing extender. The ejaculates were collected using an artificial vagina, filtered to remove gel, diluted 1:1 in prewarmed skimmed milk (isothermal condition), and centrifuged at 1,000g for 20 minutes. Postcentrifugation sperm pellets were suspended in extenders previously tempered at 20°C, packed at  $50 \times 10^6$  sperm/mL in straws of 0.5 mL, and cooled to 5°C for 90 minutes. They were subsequently exposed to liquid nitrogen vapors for 20 minutes at 4 cm over the liquid nitrogen level and finally submerged and stored in liquid nitrogen until analysis.

The cryopreservation samples were thawed at 37°C for 30 seconds before their evaluation.

In this study, only cryopreserved samples with a post-thawing sperm vitality evaluation over 40% were considered.

### 2.2. Sperm Vitality Evaluation

Vitality of the fresh sperm was evaluated using an eosin/nigrosin stain. A drop of the semen sample was mixed with a larger drop of eosin Y (2.5%), and nigrosin (5%) stains on a

prewarmed slide with an applicator stick and a thin smear was made with another slide. After air-drying, the smear was observed ( $\times 40$ ) with an LED illuminated view microscope (Nikon E200) for unstained heads of the spermatozoa (live) and stained or partially stained heads of the spermatozoa (nonviable). A total of 200 spermatozoa were counted to determine the percentage of live and nonviable spermatozoa. The average of three observations was considered a single data point.

In the laboratory, both cooled and thawed sperm samples were assessed using acridine orange (AO)/propidium iodide (PI) double staining. Sperm samples were mixed (1:1) on a tempered microscope slide with a staining solution AO/PI (20 and 10  $\mu$ M, respectively) and were immediately analyzed using the viability module from the CASA System (Sperm Class Analyzer; Microptic, Spain) and observed at  $\times 10$  with the aforementioned LED illuminated view microscope (Nikon E200). During this procedure, viable and dead spermatozoa were observed with differential fluorescent emission in the head spermatozoa (green [525 nm] for viable and red [620 nm] for nonviable spermatozoa). Viability percentages were established from a count of at least 500 spermatozoa in each sample.

### 2.3. Sodium Thioglycolate Treatment

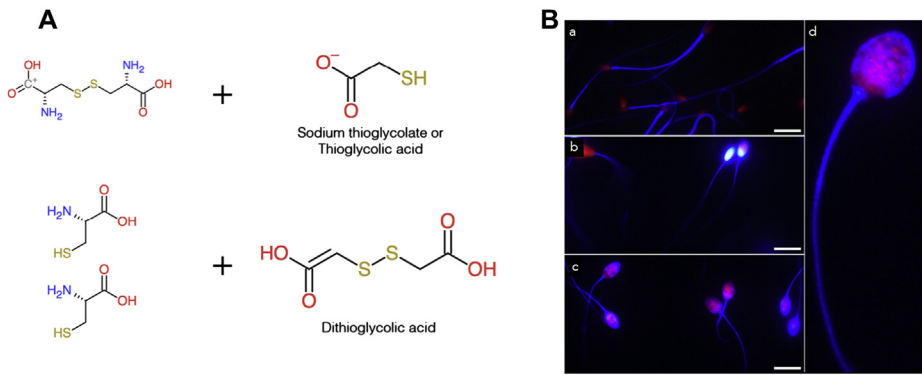
#### 2.3.1. Fornés and Bustos-Obregón (protocol A)

A volume of 1 mL of 0.4-M ST pH 9 was added to 1 mL of  $1 \times 10^4$  sperm suspension and incubated at room temperature for 15 minutes. The reaction was stopped using a 10-fold dilution with cold phosphate buffer (0.05 M, pH 7.2), centrifuged at 760g for 5 minutes, and the pellet was then resuspended in phosphate-buffered saline [5].

#### 2.3.2. Modified Protocol (B)

A number of  $1 \times 10^4$  sperm in 80  $\mu$ L of low melting point (LMP) agarose 1% was mixed at 37°C (volume of 2- $\mu$ L spermatozoa in 80- $\mu$ L agarose) and placed in 40  $\mu$ L over a 1% agarose LE glass slide prewarmed at 37°C, covered with coverslip, and left to cool for a maximum of 5 minutes at 4°C. The plasma membrane integrity was evaluated in parallel set of slides by AO/PI double staining in epifluorescent microscope.

In the not stained (AO/PI) set of slides, a drop of ST was added at different concentrations and incubated for 15 minutes (for the dose response experiment at a concentration of 0.2 mM until 200 mM) or 30 minutes (for standardized protocol, concentration 0.5 M). After incubation, the slide was dehydrated in ethanol (95% and 100%, 5-minutes each), air dried, and stained with Wright eosin methylene blue stain (Merck). The samples were stained until a layer of oxidized stain was observed (between 10 and 30 minutes), then washed with tap water, air dried, and mounted on a hydrophobic mounting medium. Resume of protocols are shown in [Supplementary Fig. 1](#). For control of reductant agent, sperm cells were incubated in DTT, 2-Mercaptoethanol (BME), and ST at 0.5, 0.05, and 0.005 M diluted in distilled water, following the protocol just indicated. This is showed in [Supplementary Fig. 2](#).



**Fig. 1.** (A) shows the reaction scheme of a disulfide bond between cysteine when sodium thioglycolate is added (draw with ChemDoodle Web Components [online], <http://web.chemdoodle.com>). The two free SH groups produced can react with reactives such as monobromobimane (mBBr) or dibromobimane (bBBr). (B) shows the SH groups created after incubation with ST being visualized with bBBr and propidium iodide staining: Control sample, without preincubation with ST (a), preincubated with ST sample (b and c), and higher magnification in (d). ST, sodium thioglycolate.

### 2.3.3. SH Group Staining by Bibromobimane

For dibromobimane (bBBr) staining, the protocol B was used with modifications, specifically, Wright staining was replaced with 2-mM bBBr (diluted in distilled water) after incubation with 10- $\mu$ M PI.

### 2.4. Nuclear Observation

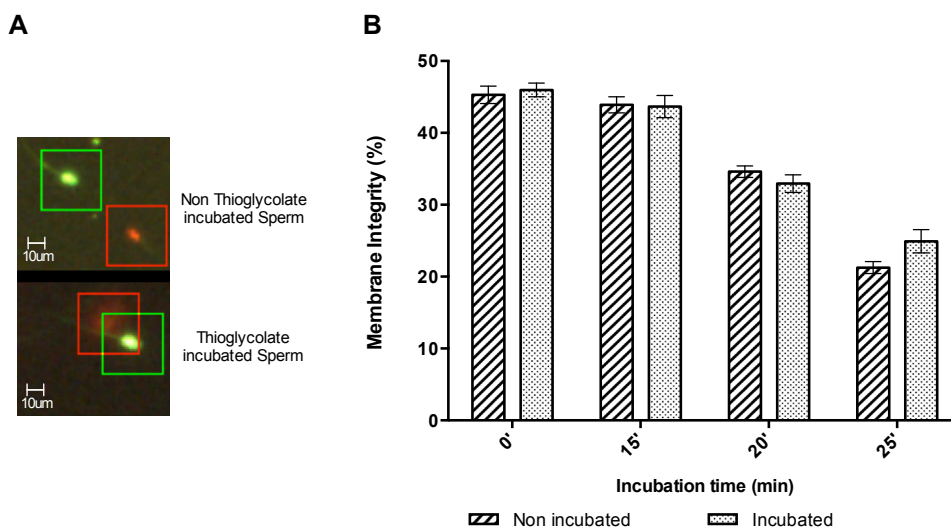
Wright eosin methylene blue staining was chosen because of its polychromatic content, where eosin stains basic cell component (e.g., protamines) and methylene blue mainly stains acid cell components (e.g., DNA). For computational analysis of protocol A, the viability module from the CASA System was used (Sperm Class Analyzer; Microptic). The size area detection (halo) was calibrated depending on signal brightness; this is due the CASA system is able to identify ranges of sizes. For our study, the

range considered for decompacted sperm was 20  $\mu\text{m}^2$ . In addition, for manual user count, a range from strong blue-stained nuclei to swelled nuclei was considered as reacted. A pale or celestial blue nuclear stain was considered as not reacted (Supplementary Fig. 2).

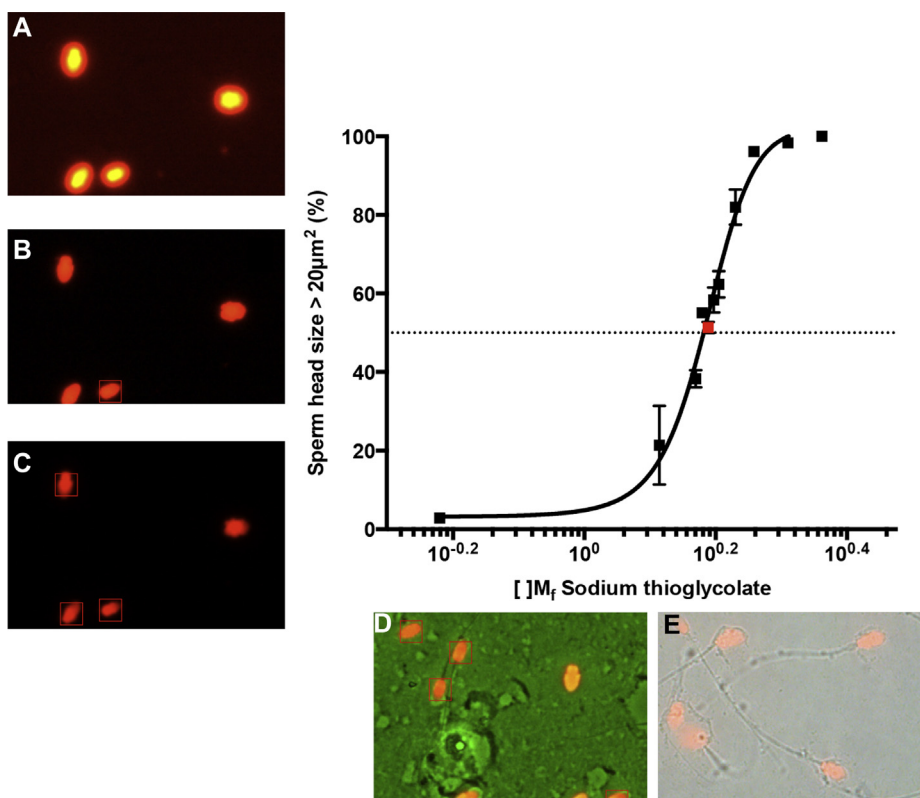
### 3. Results

The way a disulfide bond is broken and reacts with ST is shown in Fig. 1A. To check if the disulfide bond was directly affected after the ST treatment, the nuclei were observed using bBBr, as seen in Fig. 1B, in blue. The increase in the staining of bBBr and the head size after the treatment mainly is observed in Fig. 1B.

After observing decompaction following the application of protocol A, it was discovered that not all cells reacted. As a result of this finding, viability was tested



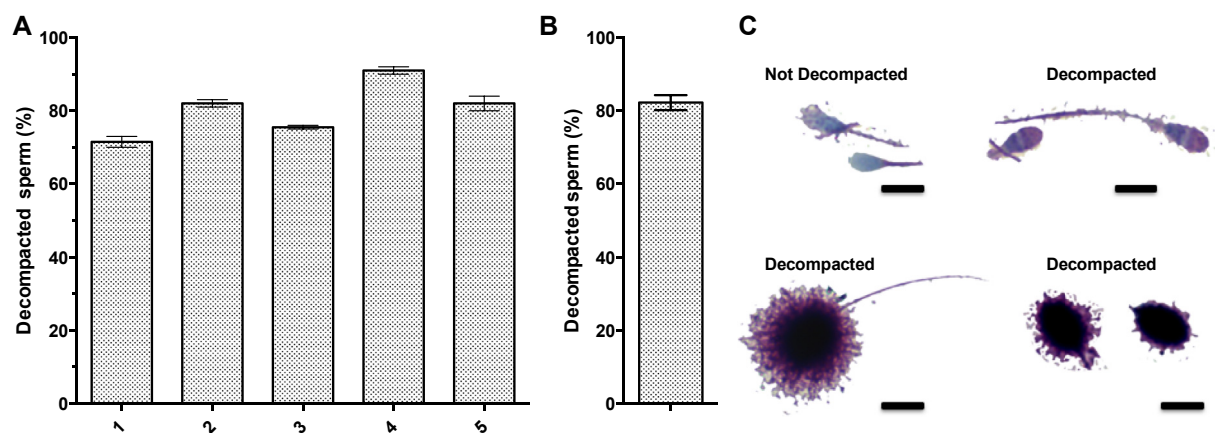
**Fig. 2.** (A) Sodium thioglycolate sperm uptake. Left, microphotography shows that only red sperm (without membrane integrity) react with sodium thioglycolate. (B) Graph shows that membrane integrity is not influenced by sodium thioglycolate incubation, and only membrane disrupted sperm suffer decondensation. Mann–Whitney test statistical analysis,  $P \leq .05$ . Plotted mean with SEM. SEM, standard error of the mean.



**Fig. 3.** Graph: Dose response of sperm head to different treatment of sodium thioglycolate. Head increase size was evaluated with Sperm Class Analyzer software, vitality module. The compacted core was defined with an area <20 µm<sup>2</sup>. (A–C) describe different configurations of the saturation field selected for analysis, pointing to a type 2 error in (A), (B) proper saturation, and a type 1 error in (C) by insufficient saturation of the image. In (D) and (E), microphotographs of stained smears of different fields with propidium iodide, combining phase contrast, and fluorescence to verify selective nuclear stain. n = 5.

after an ST treatment to determine if all the sperm in the solution had reacted. It was observed that only membrane disrupted sperm were decompacted (Fig. 2A) and that incubation time with ST did not influence the plasma membrane disruption (Fig. 2B). Not all cells reacted with ST, and reacted cells often exhibited compromised membrane integrity. This finding suggests the development of a new protocol for pretreating cells and achieving disruption. One protocol tested was to thaw

and freeze the sperm sample to disrupt the plasma membrane. Doing this twice produced cryopreserved samples with disrupted membranes, which were later incubated with the protocol A at different concentrations of ST. Fig. 3 shows that the decompaction level depends on the concentration of ST in sperm with disrupted plasma membranes, with the advantage that with these treatments, no detergent is used to produce decompaction as with SDS + DTT [7].



**Fig. 4.** (A) Comparison of reactivity to sodium thioglycolate (%) in stallion sperm samples (n = 5). (B) Mean of samples. (C) Profiles of reaction classification. Mann–Whitney test statistical analysis,  $P \leq .05$ . Plotted mean with SEM. SEM, standard error of the mean.

Moreover, it is recommended the use of ST prepared in distilled water at pH 6.2, where its redox potential is higher than DTT and 2-Mercaptoethanol (BME) ( $-281.7$  V at pH 6.9 for ST,  $-0.33$  V at pH 7 for DTT, and  $-0.26$  V at pH 7 for BME). With the disruptor pretreatment of the plasma membrane (cycles of freeze and thaw), it is not necessary to use of SDS or other detergents to facilitate the entry and action of reductant agent (Supplementary Fig. 2).

Although it was possible to count decompacted sperm cells after incubation, they tended to group with the reacted sperm on the edges of the slide and nonreacted in the center. This made counting reproducibility difficult. Thus, before treatment, the sperm cells should be attached to an agarose matrix, and later both nuclear proteins and DNA are to be treated and stained. For this purpose, protocol B is suggested, ensuring that all sperm have their membrane disrupted after the cooling process (following the addition of agarose mixed with sperm samples), and before ST treatment. With this protocol, it was possible to count the number of sperm reacted in a reproducible manner by a single individual using the CASA System. Using frozen semen samples from five stallions, the percentage reacted ranged from 77% to 97% (Fig. 4A), with an average of 81% (Fig. 4B).

Finally, considering that the reacted sperms have a head size upper to  $20 \mu\text{m}^2$ , several patterns of descompaction staining were observed (Fig. 4C).

#### 4. Discussion

In the mammal sperm nucleus, protamines are the main protein component of chromatin. However, protamines are not equally expressed between mammals [8]. In stallions, protamine one and protamine two variants have been described [9]. However, unlike for humans, where protamine one and two are present in 1:1 ratio, in stallions, the protamine two variant corresponds to 15% of nuclear protamines [10]. The different content of protamines influences their reactivity to reducing agents, which makes testing different reactants and protocols between species necessary.

No studies related to the concentration of thiol groups have been conducted for stallion sperm. However, in human sperm, protamine P1 was found to contain  $81.3 \pm 13$  mmol thiols (SH)/mol and protamine 2  $68.1 \pm 12.3$  mmol (SH)/mol [11]. Such thiol groups are readily oxidized in the air by disulfide. To maintain these groups in the reduced state, another thiol, such as cysteine, glutathione, mercaptoethanol, 2,3-dimercaptopropanol, or thioglycolate, is often added [12].

Diverse tests have been suggested to evaluate the chromatin packaging state in the sperm cell. They are all related to the disulfide bond necessary for their packing. In our case, the striking color shift of human sperm nuclei following the ST test and the observation of “halo” in treated sperm is an expected consequence of the greater availability of anionic sites [4] and the spread of basic proteins from the nuclear core; allowing us to identify compaction status of sperm cryopreserved. Moreover, the use of bBBR permitted us to identify the generation of  $-\text{SH}$  groups induced by ST pretreatment, which are related to changes in sperm head size.

In the present work, a new approximation for sperm nuclear compaction state is presented. This could be useful in the evaluation of stallion for which previous parameters did not explain their subfertile status [13].

#### 5. Conclusions

Reevaluating the chromatin compaction status with more sensitive and reproducible techniques could deliver new insights related to nuclear integrity and their relationship with sperm fertility potential. Additionally, advancements could be made relating to changes during epididymal maturation and reproducible sperm quality tests suitable for microscope software analysis.

#### Acknowledgments

With special affection and appreciation for E.B.-O. (R.I.P.). Without his guidance, friendship, and comments, the elaboration of this work would not have been possible.

This work was financed by Comisión Nacional de Investigación Científica y Tecnológica (CONICYT) Scholarship RHB and Financial Support for Doctoral Thesis Folio 21120861. Fondef CONICYT-grant D0811076.

#### Supplementary Data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jevs.2015.10.002>.

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